THE NATURAL RESISTANCE OF THE PIGEON TO THE PNEUMOCOCCUS *

WITH PLATE 7

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INTRODUCTION

Attempts to determine the biologic factors which govern natural immunity to bacterial infection have too often been attempts to reduce the mechanism of resistance to terms of a single factor. Thus, only after an extended epoch of controversy between two schools, each seeking to explain natural immunity in terms of a single general factor, namely, body cells in the one instance and body fluids in the other, have we come to recognize, as, for instance, in the rôle of opsonins, the dependence of each of these factors on the other and the fallacy of attempting to eliminate either.

In those instances, also, in which a relatively high or low body temperature has been advanced as the basis of resistance, the tendency has been to emphasize this single feature to the exclusion of other factors.

Thus, Pasteur,[1] in his early study of the resistance of fowls to anthrax, concluded that the high temperature of the fowl (41 to 42 C.) effected a direct heat-destruction of the bacteria, and that this was the full explanation of the immunity. Later, however, Hess[2] and Wagner[3] showed that phagocytosis, which occurs at the normal high temperature of the fowl, is a very important factor in immunity to anthrax, and that the increase in susceptibility produced by depressing the temperature, as practiced by Pasteur, is due, at least in part, to a reduction in the phagocytic activity of the cells at the abnormal temperature. So that in this instance also, the simpler explanation which attributed resistance to a single factor, the temperature of the body, erred in eliminating at least one other factor of importance, namely, phagocytosis.

Indeed, it may be said that in general much of the lack of success in the ultimate analysis of natural immunity appears to have resulted from the exclusion of contributing factors in the overemphasis of a single factor. Fortunately, however, the intensive study in the general field of immunity during the last two decades, has done much to correct

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[1] Bull. de l'Acad. de méd., 1878, 7, p. 140.
[2] Arch. f. path. Anat., 1887, 109, p. 365.
[3] Ann. de l'Inst. Pasteur, 1890, 4, p. 570.

this tendency and to enforce recognition of the fact that natural resistance to bacterial infection is an involved biologic reaction, not reducible to a single term, but embracing a multiplicity of factors highly interdependent and variously combined in complex and delicate adjustments. A survey of the field at this time indicates therefore that objective intensive study of individual instances of natural immunity with a view to identifying as many factors as possible there operating, may be preferred to the attempt to elevate to a position of prime importance a single factor by identifying the same in a large variety of instances.

It is the purpose of this paper to report observations concerning the distribution of pneumococci and the site of their destruction within an insusceptible host, the pigeon; and to deduce from this evidence the factors which are involved in the elimination of the bacteria, and to consider the relation of this elimination to the pigeon's high natural resistance.

Before advancing to the details of the investigation, it may be well to recall certain anatomic and physiologic features in which pigeons differ from rodents, the animals oftenest employed in laboratory experimentation.

(1) The body temperature of the pigeon is relatively high, ranging from 41 to 43 C. (106 to 109 F.). (2) The rate of metabolism is rapid. (3) The lymphatic system is relatively elementary and lacks a general distribution of lymph nodes; but nodules of lymphoid tissue are to be found in the parenchymatous organs, collected for the most part near the hilum. (4) The red corpuscles are nucleated. They are of relatively large size, measuring approximately 13 by 7 microns, and the ultimate blood capillaries are of correspondingly large caliber. (5) The white blood corpuscles conform in general to the types found in mammals, except that the cytoplasmic granules of the cells homologous to the neutrophil polymorphonuclear leukocytes of man, have the form of slender rods. These rods are often the length of two-thirds the diameter of the cell, and are closely packed throughout the cytoplasm. The affinity of these rods for acid dyes is great, and this has led to the confusing designation of the cells which they occupy as "crystalloid eosinophiles." This type of cell is not, however, in any way homologous to the type of blood cell commonly designated as the eosinophile in other species. The true eosinophile occurs in the birds also, but bears no direct relation to the so-called "crystalloid eosinophile" which, as just stated, is the homologue of the neutrophile of man. The cell under discussion is best designated as the crystalloid acidophile cell. So-called "fusiform bodies" or "ellipsoids" are present in birds' blood, closely resembling those found in amphibian blood. They appear as nuclei with a small amount of cytoplasm at either pole. The polar tabs of cytoplasm may be drawn out, or they may be blunt and vacuolated. The exact nature and the rôle of these structures are in dispute. They display a marked tendency to clump. (6) The liver and the spleen of birds contain large numbers of specialized endothelial cells, which, as I have shown

elsewhere,[4] perform the normal function of phagocytosis and digestion of red blood corpuscles. These cells, which I have designated hemophages, are shown in the accompanying drawing of the normal pigeon's liver (Fig. 1). In birds, the greater number of hemophages are active in the liver, the spleen appearing to be supplementary in this function. By virtue of a marked iron content, the cells referred to may be strikingly differentiated by Perl's Prussian-blue reaction.

The possession by pigeons of a high resistance to the pneumococcus is not in dispute. In my own experiments I have not been able, with any dose of various strains of the pneumococcus, to produce symptoms of sickness, and this accords with the results obtained by others. Explanations of this marked resistance have been numerous and varied, but for the most part based either on the high temperature of birds or on the phagocytic activity of leukocytes.

Tchistovitch[5] was among the earlier workers to place emphasis on the rôle of leukocytes in this immunity, while Strouse[6] more recently has given support to the view that phagocytosis is not a factor of importance and that the high temperature is the determining factor in the resistance of pigeons.

THE ACCUMULATION OF PNEUMOCOCCI WITHIN THE LIVER AND THE SPLEEN

A critical question in any analysis of natural immunity is that which concerns the actual site of the destruction of the bacteria, and the following experiments bear largely on this point. It was first sought to determine the distribution of injected pneumococci within the various organs and tissues of the pigeon. For this purpose 5 series of 20 pigeons each were injected by way of the leg vein with virulent pneumococci and the pigeons of each series killed in pairs after increasing intervals. The tissues were immediately fixed and later systematically examined microscopically for their content of pneumococci. The 5 series, which differed from one another only as to the number and the virulence of the organisms injected, coincided throughout as regards general results. The details here given are those relative to the group of 20 pigeons designated as Series B.

Each pigeon of this series received by way of the leg vein 2.5 c.c. of such an emulsion of pneumococci in 0.85% NaCl solution that each cubic centimeter represented the organisms washed from 4 twenty-hour blood-agar slants, the total dose in each instance representing, therefore, 10 such cultures. The particular pneumococcus employed in this series had been recovered 5 days previously from the heart blood of a man who had died of lobar pneumonia;

[4] Internat. Monatschr. f. Anat. u. Physiol., 1914, 31, p. 543.
[5] Ann. d. l'Inst. Pasteur, 1904, 18, p. 304.
[6] Jour. Exper. Med., 1909, 11, p. 743.

it had been passed once through a mouse, which it killed in 26 hours. The 20 birds thus injected were killed 2 at a time after each of the following intervals: 10 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 18 hours, 42 hours, 72 hours, 96 hours, and 112 hours. Tissue was taken at once from lung, liver, spleen, kidney, genitalia, pancreas, intestine, breast muscle, and bone marrow. The tissues were fixed in alcohol, formalin-Zenker, and Müller's fluid plus 5% mercuric sublimate; embedded in paraffin and sectioned to 4 microns. Proximal serial sections were treated with Gram's stain alone; with acid carmin followed by Gram's stain; and with Mallory's methylene blue and eosin method. The sections thus prepared from the several organs were systematically searched for pneumococci, the Mallory specimens serving as topographic controls. The findings in detail are as follows:

10-Minute Pigeons

LUNG: Pneumococci free within the small vessels. An apparent increase in leukocytes within the small vessels; no organisms seen within leukocytes. LIVER: Organisms in the capillaries in fair numbers; many apparently within cells. SPLEEN: Organisms in great numbers chiefly in association with the walls of the small arteries emerging from follicles; none within the follicles; a few in large vessels. Several cells in the pulp cords contained a few organisms each. KIDNEY: Very few organisms seen, mostly within smaller vessels and a few apparently within cells of the blood stream. PANCREAS: A few groups of 3 or 4 organisms each within the smaller arteries. INTESTINE: A few organisms within the small vessels of the villi. BONE MARROW: A few organisms throughout, apparently in close association with blood channels containing erythrocytes; some apparently contained within cells.

No organisms were found in the genitalia or the breast muscle.

30-Minute Pigeons

LUNG: Organisms frequently seen within cells of smaller blood vessels; none free in vessels. LIVER: A large number of organisms, some within cells. Number distinctly greater than at 10 minutes. SPLEEN: Groups of organisms seen along the line of small arteries; none in follicles; large number within cells. Number of organisms about the same as at 10 minutes. Many more than elsewhere except in the liver. BONE MARROW: Organisms fewer than at 10 minutes. KIDNEY: Occasional cells within small blood vessels, containing from 1 to 8 organisms; no free organisms. INTESTINE: A few organisms found in vessels of villi.

No organisms were found in the genitalia, muscle, or pancreas.

1-Hour Pigeons

LUNG: Organisms in capillaries, apparently within cells, but not all. The number about as before. SPLEEN: "Loaded" with organisms, but none in follicles, being limited almost exclusively to zone about small arteries. The few organisms in pulp cords apparently within cells. MARROW: A few organisms. LIVER: Large number of organisms within liver capillaries, apparently almost exclusively confined to cells, a single cell often containing 20 pneumococci. Distinctly more organisms than before. KIDNEY: Occasionally an intravascular cell containing from 1 to 5 organisms, but number small as compared with that found in liver and spleen. PANCREAS: A single cell with 3 organisms. INTESTINE: A few organisms in vessels of villi, all apparently within cells.

No organisms were found in genitalia or muscle.

3-HOUR PIGEONS

LUNG: Many intravascular crystalloid leukocytes, one clump of 30. Few organisms and those apparently within cells of blood stream. SPLEEN: Many crystalloid leukocytes in pulp cords. Large number of organisms throughout, most in zones about small arteries; none in follicles; many organisms apparently within cells of pulp cords. Etched and irregular forms numerous. BONE MARROW: Small number of organisms, most, apparently, within cells. LIVER: Many crystalloid leukocytes. Many organisms, for most part herded as if confined to cells of capillary walls. Many etched and irregular forms. KIDNEY: But 5 organisms seen in 5 sections and these within crystalloid leukocytes of larger vessels. PANCREAS: Three organisms found in 2 specimens.

No organisms seen in genitalia, muscle, or intestine.

6-HOUR PIGEONS

LUNG: Large number of crystalloids in capillaries. Number of organisms about the same as at 3 hours. Two crystalloid leukocytes seen with 3 and 4 organisms each. SPLEEN: Large number of crystalloid leukocytes throughout. Organisms present in goodly numbers; about the same number as at 3 hours, possibly fewer. Etched and irregular forms frequent. Almost all apparently within cells. BONE MARROW: Filled with crystalloid leukocytes. Few organisms present, for the most part within cells. LIVER: Many crystalloids in capillaries. Many organisms in clumps of 5 to 20, apparently contained within cells. Altogether slightly fewer organisms than at 3 hours. Etched and irregular forms. PANCREAS: Three crystalloid leukocytes seen, each containing from 8 to 10 organisms. INTESTINE: An occasional leukocyte seen containing 3 or 4 organisms.

No organisms seen in kidney, genitalia, or muscle.

18-HOUR PIGEONS

LUNG: Many crystalloid leukocytes in capillaries. Few organisms and these exclusively within crystalloid leukocytes. SPLEEN: Organisms fewer than previously; all within cells; frequently irregular in form and staining. LIVER: Goodly number of organisms but fewer than at 6 hours. Many surely contained within cells; the picture is distinct. Etched and granular forms are frequent. KIDNEY: Six crystalloid leukocytes found containing from 1 to 5 etched organisms each.

No organisms seen in genitalia, muscle, pancreas, intestine, or marrow.

42-HOUR PIGEONS

SPLEEN: Few organisms. All etched or granular, and apparently within cells. LIVER: Considerable number of organisms, but distinctly fewer than at 18 hours. Organisms appear to be herded within cells. Many swollen, etched, and granular forms.

No organisms found in marrow, kidney, lung, genitalia, breast muscle, pancreas, or intestine.

72-HOUR PIGEONS

SPLEEN: Few organisms, all within cells. Many swollen and etched forms. LIVER: Many organisms; but fewer than previously. Most organisms are etched and of irregular form, all apparently within cells.

No organisms seen in lung, marrow, kidney, intestine, genitalia, muscle, or pancreas.

96- AND 112-HOUR PIGEONS

No organisms seen in lung, spleen, marrow, liver, kidney, intestine, genitalia, muscle, or pancreas.

From these reports it is seen that the injected pneumococci, far from being uniformly distributed among the several organs, were preferentially deposited in the liver and spleen. Aside from liver and spleen more pneumococci were encountered in the lung and the bone marrow than elsewhere, but even in these organs, the number was in no way comparable to that observed in the liver and spleen. The approximate relative numbers of organisms present in the several organs at the various time periods are given in Table 1.

TABLE 1

THE APPROXIMATE RELATIVE NUMBERS OF ORGANISMS PRESENT IN THE SEVERAL ORGANS AT THE VARIOUS TIME PERIODS

Organ	10 Min.	30 Min.	1 Hr.	3 Hr.	6 Hr.	18 Hr.	42 Hr.	72 Hr.	96 Hr.	112 Hr.
Lung.....	++	++	++	+	+	+	0	0	0	0
Spleen.....	+++	+++	++++	++++	+++	++	+	+	0	0
Bone marrow..	+	+	+	++	+	0	0	0	0	0
Liver.....	++++	+++++	++++ ++++	+++ ++++	++ ++++	++++	+++	++	0	0
Kidney.....	+	+	+	0	0	+	0	0	0	0
Genitalia.....	0	0	0	0	0	0	0	0	0	0
Breast muscle..	0	0	0	0	0	0	0	0	0	0
Pancreas.....	+	0	+	+	+	0	0	0	0	0
Intestine.....	+	+	+	0	+	0	0	0	0	0

An analysis of Table 1 shows that at all the intervals from 10 minutes to 72 hours the liver contained many more pneumococci than did any other organ. The spleen ranked next, tho showing distinctly fewer pneumococci than the liver had shown. In other organs the numbers were relatively inconsiderable. The contrasts recorded were marked far beyond the fine quantitative differences such as might be caused by accidental variations in distribution, or by differences between the particular parts of the organs from which specimens were taken. Moreover, as stated previously, 4 similar series of inoculations gave corresponding results.

The rate of the differential localization of pneumococci is rapid; as early as 10 minutes after injection the accumulation of pneumococci in the liver and in the spleen was already distinctly apparent. The rate

of destruction of pneumococci within these organs is also rapid; after 72 hours both the liver and the spleen were found to be free of the organisms.

The significance of these findings is in the fact that the completeness of the accumulation of the pneumococci in the liver and the spleen, the promptness with which the localization occurs, and the rapidity of the destruction of the organisms so segregated, constitute an actually efficient process for the elimination of pneumococci from the circulating blood.

THE MECHANISM OF THE ACCUMULATION OF PNEUMOCOCCI WITHIN THE LIVER AND SPLEEN

The recognition of an extensive accumulation in the liver and the spleen of pneumococci introduced into the general blood stream, involves at once the question as to the mechanism by which this accumulation is effected. A histologic study of these organs has led me to the conclusion that phagocytosis by fixed tissue cells is the basis of this accumulation, to which, in the spleen, is added a supplementary factor in the filtering action of the modified vascular wall of an artery peculiar to that organ.

In the reports reference is made to the fact that the pneumococci within the liver and the spleen were often observed to be in considerable aggregates, as tho herded within the confines of a cell. Nevertheless, with the more usual histologic methods, it still remained a question whether the pneumococci were actually intracellular, and if so, in what type of cell they were included. To obtain more decisive morphologic evidence in this regard I developed a method which is essentially a combination of Perl's Prussian-blue reaction for iron, of Gram's stain, and of one or more counterstains. The introduction of the Prussian-blue reaction was suggested by the fact that the bacteria, where collected in clumps, were in close relation with masses of golden-yellow pigment. This pigment appeared the same as the iron-containing pigment which I have elsewhere shown to be constantly present within certain endothelial cells of the liver and the spleen of normal pigeons, and which results from an intracellular digestion of red blood corpuscles by those cells (hemophages). Moreover, in the normal pigeon, the iron-containing pigment when subjected to Perl's test sharply differentiates the containing cell by virtue of the Prussian-blue formed within the cell body. In view of these facts it appeared possible that

in the case of the injected pigeons, the close relation of pigment and bacteria might be due to the inclusion of both within the same cell, and that this relation might be made apparent by the sharp differentiation of the containing cell by the Prussian-blue reaction. Such proved to be the case and the method was most extensively employed according to the following formula:

Fix thin slices of tissue for from 18 to 24 hours in Müller's fluid plus 5% mercuric sublimate. Imbed in paraffin and section to 4 microns. Fix sections upon slide and immerse 10 minutes in equal parts of a 2% aqueous solution of potassium ferrocyanid, and of a 2% aqueous solution of hydrochloric acid, sufficient sodium chlorid being added to bring the combined solution up to 2%. Wash in 2% sodium chlorid and stain for 20 minutes in acid carmin. After washing hurriedly in 50% alcohol, stain by Gram's method, decolorization being effected in a mixture of toluol (2 parts) and anilin oil (1 part). Wash in toluol, and mount in Canada balsam.

Prepared by this method, the liver and spleen of the injected pigeons afforded a striking picture. As in the case of the liver and the spleen of normal pigeons, both the organs showed an extensive content of clearly differentiated cells possessing the distinct blue-green tone of the Prussian-blue iron reaction. These hemophages were somewhat more numerous, or at least more prominent, in the case of the injected birds than in normal birds. The cells were of the same type in both organs and in the liver were clearly recognizable as vascular endothelium of the venous capillaries. In the spleen the vascular relation was less evident, and no hemophages were found within the follicles; in other words, they were confined to the pulp cords. Wherever present, the hemophages contained ingested red blood corpuscles, or products of their digestion, and in the liver the inclusions produced a marked bulging of the cells into the lumen of the vessel. From the point of view of the present investigation, however, the most striking phenomenon observed in such specimens was the inclusion by the hemophages of practically all pneumococci injected (Figs. 2 and 3). With the sharp differentiation of the cell body of the hemophages by the Prussian-blue reaction, all doubt concerning the intracellular presence of the organisms was eliminated. Pneumococci were seen in most of the hemophages, and in a given hemophage in great numbers. In the 4-micron section of a hemophage, not infrequently 50 pneumococci were to be counted, this indicating that the content of the total cell was several hundred organisms. In many cells the organisms were distributed rather uniformly throughout the cytoplasm, while in others distinct vacuoles containing from 10 to 30 pneumococci were frequently

observed. The contained organisms displayed in many instances morphologic changes resulting from intracellular digestion—etched, swollen, and granular forms being the most frequent.

With the fact established that the hemophages of the liver and the spleen so extensively take up pneumococci, a detailed survey was made of tissue of these two organs taken from all pigeons of Series B, with especial reference to the relative numbers of the organisms actually contained within the hemophages. The results showed that throughout the series practically all pneumococci accumulated within the liver were contained within the hemophages. Occasionally 1 to 3 pneumococci could be found apparently free in a lumen or within a crystalloid acidophile leukocyte of the circulating blood, but this occurrence was rare and limited almost exclusively to pigeons killed within one-half hour of their injection. Even in such instances, however, the number of organisms not included within hemophages would not, in a total averaged-sized microscopic preparation, equal the number of organisms often seen within a single hemophage of the same specimen.

It thus appears that the marked accumulation of pneumococci in the liver subsequent to their intravenous injection is due exclusively to the extensive engagement of the organisms by that type of fixed phagocyte which has for its normal function the destruction of red blood corpuscles.

In the spleens of the series, the hemophages were seen to play the same rôle as in the liver, the pneumococci ultimately being accumulatd within these cells and there digested. Cell for cell, the hemophages of the spleens contained as many pneumococci as did those of the livers. The absolute number of hemophages was, however, as in normal pigeons, distinctly smaller. Moreover, the primary localization of the pneumococci within the spleens was not due, as in the livers, exclusively to the action of the hemophages. A mechanical filtration of the organisms by the walls of certain of the blood vessels was found to be an important factor in the rapid accumulation of the pneumococci within this organ, and the structure of these vessels therefore requires consideration in detail. The small vessels emerging from the follicles of the bird's spleen consist of a delicate endothelial intima surrounded by a double or even triple layer of spherical cells sharply differentiated from other tissue elements and supported in a delicate reticulum meshwork. This double or triple layer of cells constitutes a broad zone about the lumen of the vessel and the wall thus constituted bears but slight resemblance to any other vessel wall with which I am acquainted.

The sharpness of the demarcation of the outer margin of the layer, the uniformity of its area of cross section, and the characteristic staining reaction of the constituent cells reveal it, however, as a vascular coat supporting and supplementing the intima of the vessel in question. In cross section this vascular coat appears of relatively great thickness but of loose texture. The general structure of such vessels in cross section is shown in Figure 4.

With this brief statement concerning the structural detail of the splenic vessels in question, it may be stated that they serve a distinct function as filters under the conditions of the experiments here recorded, and operate to accumulate promptly intravascular pneumococci within the spleen. The evidence for this is seen in the distribution of the pneumococci within the spleen soon after injection. In the 10-minute spleens, for instance, practically all pneumococci observed are found between the cells of the vascular coat. No organisms are seen in the follicles and but very few are at this time found within the hemophages of the pulp cords. In a 4-micron section of a single vessel of this type, 10 minutes after injection, it is frequently possible to observe from 50 to 100 pneumococci definitely limited to the vessel wall (Fig. 4). The pneumococci are not intracellular. They are distinctly interstitial and are not clumped. The vascular zone is free of hemophages as are also the follicles. At this period a few intravascular crystalloid leukocytes may be found containing from 1 to 4 pneumococci and an occasionally free pneumococcus may also be seen in the blood stream. In 30-minute spleens the number of pneumococci within the zones is much increased, but a considerable number of organisms also have emigrated to the pulp cords in the vicinity of the zones, and have been engulfed by hemophages there contained. In 1-hour spleens also, there are many pneumococci between the cells of the vessel walls, but, in addition, they are now found widely distributed throughout the pulp cords, being contained within hemophages. In spleens from 3-hour and 6-hour pigeons the findings contrast with those in the earlier animals in that the greater proportion of organisms is now found within the hemophages of the pulp cords, and relatively few are retained within the vascular zones. In the pigeons killed 18 hours or longer after injection, the zones are free from pneumococci, the organisms having been transported completely through the vessel wall and engulfed by hemophages of the pulp cords.

Surveyed from the point of view of function this distribution of the pneumococci within the spleen after various intervals leads to the conclusion, (1) that pneumococci which enter the spleen by way of its closed vascular system rapidly leave this system, being transported into, and eventually through, the loose vascular wall of certain modified arterioles whose interstices allow the free outward passage of plasma but not that of the formed elements of the blood; (2) that the interstices between the cells constituting the great bulk of the vascular wall in question are of such minute size as to allow but a retarded transportation of pneumococci through them, the tissue thus operating as a partial filter accumulating the organisms within its area; and (3) that having thus gradually been washed through the vessel wall, the pneumococci are brought into contact with the hemophages of the pulp cords and by these are ingested and digested as in the liver. Thus, vast numbers of pneumococci are rapidly filtered from the plasma by a mechanism peculiar to the spleen, to be ultimately ingested and destroyed, however, by the same kind of phagocytic cell that we find in the liver. In both organs, therefore, the ultimate localization and destruction of the injected organism is within fixed phagocytes.

THE RELATION OF FIXED PHAGOCYTOSIS TO THE IMMUNITY OF THE PIGEON

I have shown that pneumococci introduced into the blood stream of pigeons are rapidly localized in the liver and the spleen and are there destroyed within fixed phagocytes. This demonstration does not prove that the immunity of the pigeon is based on this phagocytosis alone. The extent of the process, however, does indicate that this phagocytosis is a factor of great importance and possibly even the determining factor in the immunity. At best the degree of this importance can be determined only approximately, in view of the incompleteness of our present knowledge as to the many additional factors which may contribute to resistance. But so far as an estimate can be made, it is to be gained from a comparison of the efficacy of the fixed-tissue phagocytosis with that of such factors as wandering-cell phagocytosis, the antibacterial properties of the body fluids, and the influence of the temperature of the host.

In making such a comparison it should be borne clearly in mind, relative to fixed-tissue phagocytosis, that the significant feature is not the determination that a phagocytosis occurs but rather the demonstration of the great rapidity and extent of its occurrence. It is not diffi-

cult to conceive a fixed-tissue phagocytosis which, altho demonstrable, might involve so few organisms or proceed so slowly as to be practically negligible with respect to the elimination of the infecting organisms. In the present instance, however, both the extent and the rapidity of the phagocytosis are extreme: not a few, but practically all pneumococci introduced into the blood stream are taken up by the hemophages and this within a few hours. The phagocytosis, moreover, is a phagocytosis of living organisms, as shown by the fact that the liver is highly infectious up to 6 hours after injection, even after vascular washing to the point where the fluid recovered is slightly, and at times not at all, infectious. It is with such a phagocytosis that other factors must be compared.

Wandering-cell phagocytosis is clearly not a considerable factor in the immunity of the pigeon to the pneumococcus. This point I wish to emphasize the more because of the frequent confusion of fixed phagocytes with wandering cells, and because of the rather general conception that phagocytosis in relation to immunity coincides with phagocytosis on the part of wandering cells. Thus, Metchnikoff[7] expressed the view that the Kupffer cells — the hemophages of the liver — are to be regarded as wandering cells, namely, phagocytic white blood corpuscles which are somewhat delayed in the capillaries of the liver. The sharp differentiation of these cells in the pigeon's liver which I have obtained with the Prussian-blue reaction, shows conclusively, however, that these cells are not leukocytes but fixed cells. Their phagocytosis therefore is not to be confused with that accomplished by wandering cells.

The injection of pneumococci into the blood stream of pigeons produces a leukocytosis. This leukocytosis is almost exclusively an increase in the crystalloid acidophiles — the homologues of the neutrophiles in man. The normal average leukocyte count in pigeons is approximately 10,000 per cubic millimeter and there is usually no increase in this number for 1 hour after injection of pneumococci. In from 1 to 2 hours after injection, however, there is the commencement of a very sudden increase in the leukocytes, which may result in a content of 40,000 per cubic millimeter during the fourth or fifth hour. Between the tenth and twentieth hours from 50,000 to 100,000 per cubic millimeter is common. Soon after this period, however, the leukocytosis decreases rapidly so that at 40 hours, or even earlier,

[7] L'immunité, 1901, p. 80.

the leukocyte count is that of the normal animal. This cycle is the one observed following an intravenous injection of living virulent pneumococci.

Concerning the occurrence of a marked leukocytosis, therefore, there is no doubt; but equally striking is the almost complete lack of phagocytosis by the leukocytes. The results obtained both from blood smears and from the fixed tissues taken at various periods, show that of the vast number of leukocytes present, but very few contain pneumococci and then only in small numbers. (Unless the decolorization in Gram's method is extensive, the rodlike granules of the crystalloid acidophiles may so far simulate bacteria as to lead to the interpretation of phagocytosis when none exists. No such confusion occurs, however, when the extraction of the gentian violet is accomplished by a mixture of toluol and anilin oil.

Altogether the phagocytosis by wandering cells is very slight, and compared with the phagocytosis by the hemophages is so insignificant as to be negligible as a factor in the destruction of pneumococci and in the resistance of the host.

The evaluation of the rôle of the body fluids in the resistance of the pigeon to pneumococci can not be made with definiteness. Within the host no direct determination of the antibacterial action of these fluids can be effected apart from many complicating factors, and the analyses obtained in vitro carry no guarantee that the properties displayed there are more than remotely related to those which actually obtain within the animal. So far as in-vitro experiments are of value, however, they give no evidence of a distinct antibacterial action on the part of the blood serum — a conclusion reached also in the work of Strouse.[6]

Pneumococci subjected to the action of the serum in vitro do not show a diminished viability or pathogenicity beyond that produced by similar treatment with the sera of highly susceptible species. In fact, on agar slants with 2 c.c. of fresh serum at the bottom of the tube, the growth of pneumococci is most abundant at the line of contact betwen the serum and the agar; and pneumococci injected into mice 2, 4, and 8 hours after suspension in pigeon's serum show as great a killing power as when suspended similarly in salt solution or in the sera of the susceptible species—mouse, man, and rabbit.

In regard to the opsonic power of pigeon's serum there is also no evidence of any marked action, and here again my results coincide with

those of Strouse.[6] The lack of any considerable phagocytosis of injected pneumococci by the leukocytes in the circulating blood stream, in itself, shows the absence of an opsonizing power, effective in relation with these cells. Tests in vitro of the opsonic value of the serum in relation to the pigeon's own leukocytes are, in my opinion, of no value whatever because of the extreme fragility of these leukocytes when removed from the animal. (So delicate are the crystalloid acidophile leukocytes that, removed from the body, they rapidly go to pieces even in the pigeon's own serum. They cannot be suspended in the usual diluents employed in leukocyte-counting, but for this purpose must be preserved by rapid fixation, preferably in warm osmic acid. Those structures counted as leukocytes in the usual determinations of bird's blood are for the most part nuclei of hemolysed erythrocytes; hence the great discrepancies between the figures of various workers—ranging from 10,000 to 50,000 per cubic millimeter.) Tested with the leukocytes of other species (mouse, man, guinea-pig, and rabbit), pigeon's serum shows no definite opsonic power. Altogether, then, such results as have been obtained by test-tube methods fail to show that the blood serum of the pigeon possesses a peculiar content of antibacterial substances which might operate to determine the immunity under discussion.

The relatively high temperature of the pigeon requires careful consideration. Influenced by the early work of Pasteur on fowl anthrax, and by the fact that the temperature of pigeons coincides approximately with the maximal surviving temperature of the pneumococcus on artificial media, a school of workers has contended that the immunity of that animal is due to a direct heat-destruction of the bacteria within the host. In support of this view investigations have been advanced to show that phagocytosis is lacking, and the deduction made that because of this lack, heat-destruction is the presumable factor which confers the resistance. The most explicit data in support of this view are those furnished in the work of Strouse.[6] In regard to this contention, the determination which I have made of an extensive fixed-tissue phagocytosis possesses a direct bearing. The results of my experiments show not only that phagocytosis is not lacking, but that its occurrence is of such magnitude as to demand its consideration as the major factor contributing to the pigeon's immunity.

As indicated at the commencement of this article, I do not, in emphasizing the rôle of phagocytosis, wish to attempt to exclude possible con-

tributing factors, and among them temperature. On the other hand, the exalted position given temperature on the basis of negative findings as to phagocytosis is most certainly invalidated by the present demonstration that an extensive phagocytic destruction of pneumococci does occur, and further considerations relative to this phagocytosis indicate that the influence of the high temperature is at most subsidiary and indirect.

Among such considerations is the fact that the phagocytic destruction of the bacteria is accomplished in distinctly less time than that required for heat destruction at the maximal temperature of the pigeon. Ninety-six hours at 43 C. does not destroy pneumococcus cultures, but, injected into the blood stream of the pigeon, several billions of pneumococci are taken up within 10 minutes, and totally engaged and destroyed in about 72 hours.

A second indication that the resistance of the pigeon is not primarily due to high temperature is seen in the fact that 2 strains of pneumococci acclimated to 42.4 C. for several months, showed no pathogenicity or increased viability when injected into pigeons, altho rendered lethal for white mice and rabbits by passage. Their phagocytic destruction was in all ways similar to that of the usual cultures grown at 38 C.

The chief contention which I wish to make, however, is not that the temperature of the body, or indeed the action of body fluids, may be eliminated from all consirderation as contributory factors, but rather that phagocytosis does occur, and occurs in such magnitude as to demand its acceptance as a factor of major, if not determining, importance in the immunity displayed by the pigeon to the pneumococcus.

In the bulk of my experiments the organisms were introduced directly into the circulating blood stream. Were the action of the hemophages confined to the destruction of organisms so introduced. the general significance of their activity in the total immunity of the host might well be considered limited. This is not the case, however, for subsequent to their inoculation by other channels, pneumococci rapidly gain entrance to the blood stream, and, borne to the hemophages, are destroyed as are those injected intravenously. In the case of intraperitoneal injections the rapidity with which the organisms reach the general circulation, and thereby the hemophages, is most striking.[8] Whether it be the unusual size of the lymphatic spaces and vessels leading from the peritoneal cavity, or the absence of interposed nodes in their course, has not been determined, but the fact is established that

[8] Berry and Melick: Jour. Immunol., 1916, 1, p. 47.

pneumococci injected into the abdominal cavity reach the hemophages of the liver and spleen, and are there destroyed, in a period but slightly greater than that occupied in the destruction of organisms introduced directly into the blood stream.

CONCLUSIONS

In the pigeon, a species insusceptible to the pneumococcus, the infecting organisms are rapidly withdrawn from the general blood stream and localized in the liver and the spleen.

In both these organs the ultimate localization of the pneumococci is within a type of fixed phagocyte—the hemophage—common to both organs, and having for its normal function the destruction of red blood corpuscles.

This phagocytic destruction of the pneumococci by hemophages is so extensive and so rapid as actually to constitute an important, if not indeed the determining, factor in the establishment of this instance of natural immunity.

EXPLANATION OF PLATE 7

FIG. 1. Section of the normal liver of a pigeon showing differentiated hemophages. I=nuclei of hemophage. II=ingested erythrocyte. Camera lucida. Zeiss ocular 4, objective 2 mm. oil. × 820.

FIGS. 2 AND 3. Sections of the livers of pigeons killed one-half and one hour, respectively, after injection of pneumococci. Content of ingested pneumococci within hemophages. I=nuclei of hemophages. II=ingested erythrocytes. III=nuclei remnants of digested erythrocytes. Camera lucida. Zeiss ocular 8, objective 2 mm. oil. × 1600.

FIG. 4. Section of the spleen of a 10-minute pigeon showing pneumococci accumulated within the vascular zones. Camera lucida. Zeiss ocular 6, objective 2 mm. oil. × 1050.

PLATE 7